EXHIBIT C

Intercellular delivery of functional p53 by the herpesvirus protein VP22

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The herpes simplex virus type 1 (HSV-1) virion protein VP22 exhibits the remarkable property of int r-cellular trafficking whereby the protein spreads from the cell in which it is synthesized to many surrounding cells. In addition to having implications for protein trafficking mechanisms, this function of VP22 might be exploited to overcome a major hurdle in gene therapy, i.e., efficient delivery of genes and gen products. We show that chimeric polypeptides, consisting of VP22 linked to the entire p53 protein, retain their ability to spread between cells and accumulate in recipient cell nuclei. Furthermore the p53-VP22 chimeric protein efficiently induces apoptosis in p53 negative human osteosarcoma cells resulting in a widespread cytotoxic effect. The intercellular delivery of functional p53-VP22 fusion protein is likely to prove beneficial in therapeutic strategies based on restoration of p53 function. These results, demonstrating intracellular transport of large functional proteins, indicate that VP22 delivery may have applications in gene therapy.

Keywords: protein engineering, protein delivery, gene therapy

The introduction of nucleic acids (including genes, antisense RNAs, or ribozymes), peptides/proteins, and small molecules into the appropriate target cells and tissues is being developed as a therapeutic approach to a range of diseases. Much progress has been made in our understanding of the molecular biology of cell division and differentiation, the identification of disease susceptibility genes, and the ability to develop high throughput screens for antagonists or agonists of particular targets. A remaining problem inherent to all aspects of gene therapy lies in the delivery of the therapeutic agent to a sufficiently high percentage of cells in sufficient and appropriate amounts to elicit a sustained therapeutic response[1].

The herpes simplex virus type 1 (HSV-1) tegument protein VP22 exhibits the remarkable property of intercellular spread whereby this 38 kDa protein is exported from the cytoplasm of an expressing cell and subsequently imported into many neighboring cells where it accumulates in the nucleus[2]. The precise mechanism of this transport is not currently understood, but is believed to be via a Golgi independent pathway. This property of intercellular spread of VP22 might be exploited in the development of gene therapy delivery systems.

Considerable effort has been directed to cancer gene therapy using *p53*, a gene that is mutated in a wide range of human malignancies[3]. p53 regulates cell cycle progression and, under conditions of DNA damage, through a complex signal transduction pathway, can induce cell cycle arrest or apoptosis[3]. Failure to synthesize p53 or, more commonly, synthesis of a mutated form of the protein results in uncontrolled cell proliferation and tumor formation.

We demonstrate that VP22-p53 chimeric proteins (approximately 90 kDa) retain the ability to spread between cells. Furthermore, these fusion proteins retain the ability to induce apoptosis in a human osteosarcoma cell line. These results indicate that enhanced delivery of not only peptides and small proteins, but also of large multifunctional proteins by VP22 may be achieved and may be applicable in a wide variety of therapeutic arenas.

Results and discussion

To investigate the ability of VP22 to transport p53 protein between cells, we constructed expression vectors for full-length, in-frame VP22-p53 and p53-VP22 fusion proteins, driven by the cytomegalovirus immediate-early enhancer/promoter region (Fig. 1A). The chimeric proteins also contain an epitope tag at their C-terminus, the detection of which ensures full-length synthesis. After transfection into COS-1 cells, both vectors generated predominantly full-length fusion protein of approximately 90 kDa, which were detected by Western blot analysis using anti-VP22, anti-p53, and anti-epitope antibodies (Fig. 1B–D).

To determine whether the VP22-p53 and p53-VP22 fusion proteins were able to spread from a primary expressing cell into neighboring cells, monolayers of cells transfected with wild type VP22 or the fusion protein constructs were examined by immunofluorescence staining using each of the antibodies (Fig. 2).

In control experiments the anti-VP22 antibody showed no cross-reaction with mock transfected cells (Fig. 2A). Intercellular transport was clearly observed in wild type VP22 transfected cells (Fig. 2B, anti-VP22 antibody) where the primary expressing cells contain VP22 largely in the cytoplasm and surrounding recipient cells accumulate VP22 in their nuclei[2]. Endogenous nuclear p53 protein could be detected with the anti-p53 antibody (Fig. 2C). As expected the anti-epitope antibody showed no cross-reaction with mock transfected cells (Fig. 2D).

In cells expressing VP22-p53 (Fig. 2E–J), detected using anti-VP22 antibody (Fig. 2E), a pattern of localization representing intercellular transport was clearly observed, with more intensely staining cells in which the fusion protein was largely cytoplasmic (long arrows), surrounded by numerous recipient cells in which the fusion protein had accumulated in the nucleus (short arrows). An identical pattern of localization was observed when these cells were labeled with an anti-p53 antibody (VP22-p53, Fig. 2F). Unlike the anti-VP22 antibody, the anti-p53 antibody detects endogenous p53 in addition to the VP22-p53 fusion. To avoid this and at the same time ensure that the transport detected by anti-

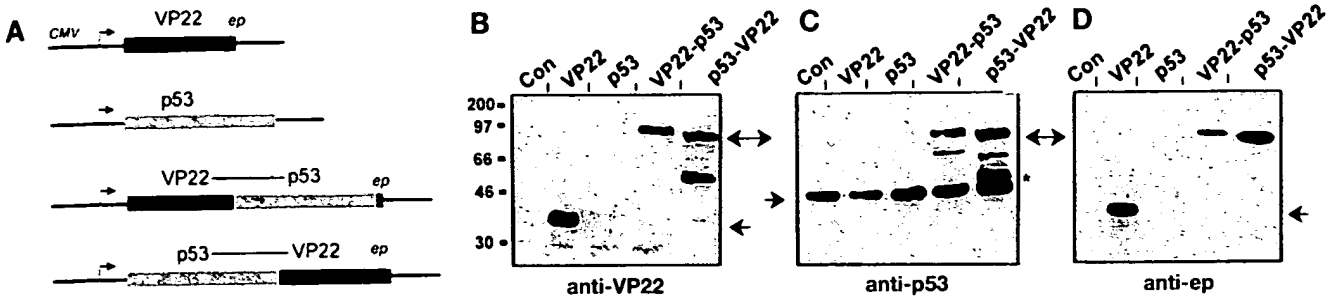

Figure 1. Expression of VP22-p53 fusion proteins. (A) Schematic illustration of the expression vectors encoding wild type VP22, wild type p53, and the two epitope-tagged p53 fusion proteins VP22-p53 and p53-VP22, each under the control of the cytomegalovirus IE promoter. Extracts from COS-1 cells transfected (2 µg DNA) with pUC19 (Con), or expression vectors for w/t VP22 (VP22), wild type p53 (p53) VP22-p53 and p53-VP22 were separated by SDS electrophoresis, blotted and probed with antibodies against (B) VP22, (C) p53, or the (D) epitope tag. VP22 is indicated by leftward arrows, wild type p53 by rightward arrows, and the fusion proteins by double-headed arrows. Fusion protein p53-VP22 appears to be fractionally smaller than VP22-p53 on SDS-PAGE and is more susceptible to clipping at the C-terminus, indicated by the asterisk.

VP22 antibody was indeed full-length fusion protein, VP22-p53 transfected cells were simultaneously labeled with anti-VP22 and anti-epitope antibodies (Fig. 2G and H). The fusion protein was detected by both antibodies, confirming that the full-length protein was transported. The relative efficiency of transport of the VP22-p53 fusions appeared only slightly less than wild type VP22, as estimated from the numbers of VP22 or fusion protein positive cells in isolated foci. Identical results were obtained with the chimeric protein in which the p53 protein was fused to the N-terminus of VP22, (data not shown).

To confirm intercellular transport, COS-1 cells transfected with the VP22-p53 expression vector were trypsinized 24 h after transfection and mixed at a ratio of 1:20 with nontransfected Vero cells. The mixed cell population was incubated for another 24 h. The co-plated cells were then simultaneously stained for T-antigen (to detect the COS-1 cells, which are uniformly T-antigen positive) and the fusion protein (Fig. 2I and J). The fusion protein was detected not only in T-antigen-positive COS-1 cells but also was readily detected in the surrounding T-antigen-negative Vero cells, further demonstrating spread of the fusion proteins.

Extensive evidence suggests that a key feature in tumor progression is likely to be in the escape from cell cycle checkpoint control and induction of apoptosis effected by normal *p53* in response to DNA damage. To assay for p53 function of the VP22 fusions we used the *p53*-negative osteosarcoma cell line, SAOS-2. These cells harbor a homozygous deletion at the *p53* gene locus and do not produce p53 protein[4].

Reintroduction of *p53* suppresses the growth of SAOS-2 cells, leading to the induction of apoptosis[5]. SAOS-2 cells were either mock transfected or transfected with plasmid vectors expressing wild type VP22, wild type p53, VP22-p53, or p53-VP22 (Fig. 3). The cells were stained with crystal violet 48 h after transfection and examined under low power magnification to assess the integrity of the monolayers. For each transfected monolayer cells in numerous random fields were enumerated and cell counts expressed as a percentage of the mock transfected monolayer. Transfection itself had an effect on the SAOS cells as a control monolayer grown without any change of buffers or transfection had slightly greater cell numbers than the pUC19 transfected cells, (data not shown). There was, however, no significant

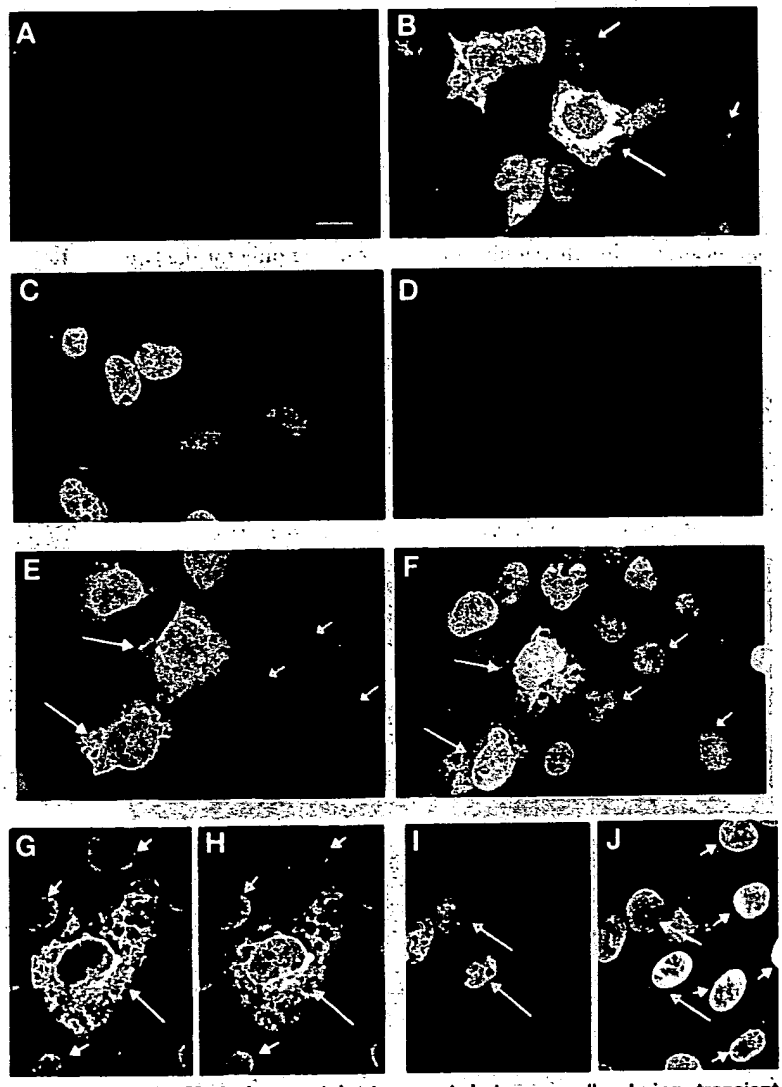

Figure 2. VP22-p53 fusion protein transport between cells during transient expression. Indirect immunofluorescent staining of COS-1 cells transfected as a control with pUC19 labeled with anti-VP22 (A), anti-p53 (C) and anti-epitope antibodies (D), or (B) transfected with wild type VP22 and labeled with anti-VP22. COS-1 cells expressing the C-terminal VP22-p53 fusion protein were double-labeled with (E) anti-VP22 and (F) anti-p53 antibodies or (G) anti-VP22 and (H) anti-epitope antibodies and detected by indirect immunofluorescence. Primary expressing cells are labeled with long arrows, recipient cells with short arrows. COS-1 cells transfected with VP22-p53 were mixed with untransfected Vero cells at a ratio of 1:20 and double-labeled for (I) T-Antigen and (J) VP22. Expressing COS-1 cells are labeled with long arrows, recipient Vero cells with short arrows. Bar represents 10 µm.

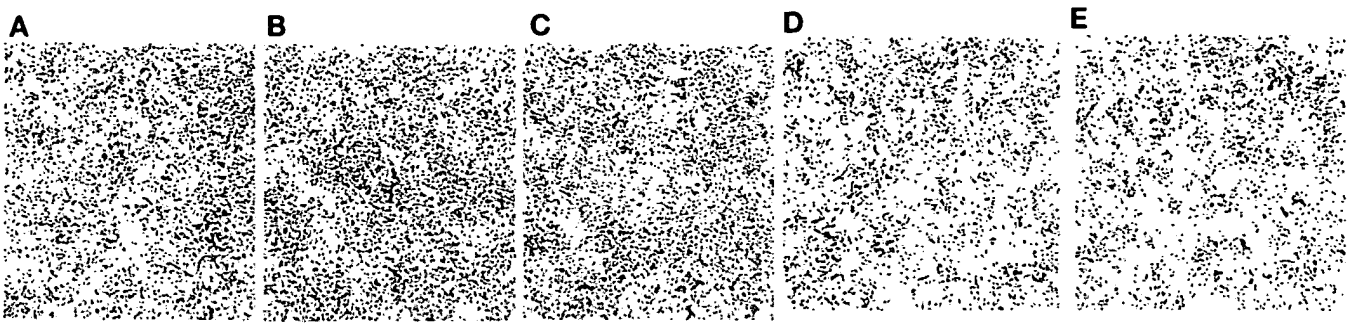

Figure 3. Effect of VP22-p53 on SAOS-2 cells. p53-negative SAOS-2 cells were (A) mock transfected or (B) transfected with vectors expressing wild type VP22, (C) wild type p53, (D) VP22-p53, or (E) p53-VP22. Cells were fixed 48 h posttransfection and stained with crystal violet to assess the integrity of the monolayer.

difference between the control transfected cells (Fig. 3A) and cells transfected with wild type VP22 (Fig. 3B). It was difficult to determine an effect of wild type *p53* by this assay (Fig. 3C), and no significant difference was observed in cell counts in the *p53* versus the mock transfected cell monolayers, presumably reflecting low transfection efficiency. But induction of apoptosis by *p53*, above that of control levels was observed (see below). However, expression of VP22-p53 or p53-VP22 resulted in a loss of $32 \pm 5\%$ and $41 \pm 7\%$ of the cell monolayer, respectively (Fig. 3D and E), relative to mock transfected and control monolayers. This represents a statistically significant loss in cell viability and was observed only for the two VP22 fusion proteins, consistent with a more widespread cytotoxic effect in both primary expressing cells and cells that have obtained the fusion protein by cell to cell spread.

To examine further the cytotoxic effect of the VP22-p53 fusion proteins in SAOS-2 cells and to confirm that these cells can support spread of VP22, COS-1 cells were transfected with the VP22-p53 expression construct, the VP22 parental construct or wild type p53 as a control, then trypsinized and co-plated with untransfected SAOS-2 cells. T-antigen-positive wild type VP22 transfected COS cells (Fig. 4A) demonstrated spread of wild type VP22 into adjacent SAOS-2 cells (Fig. 4B) with no apparent deleterious effect on the monolayer. Endogenously p53-positive COS-1 cells transfected

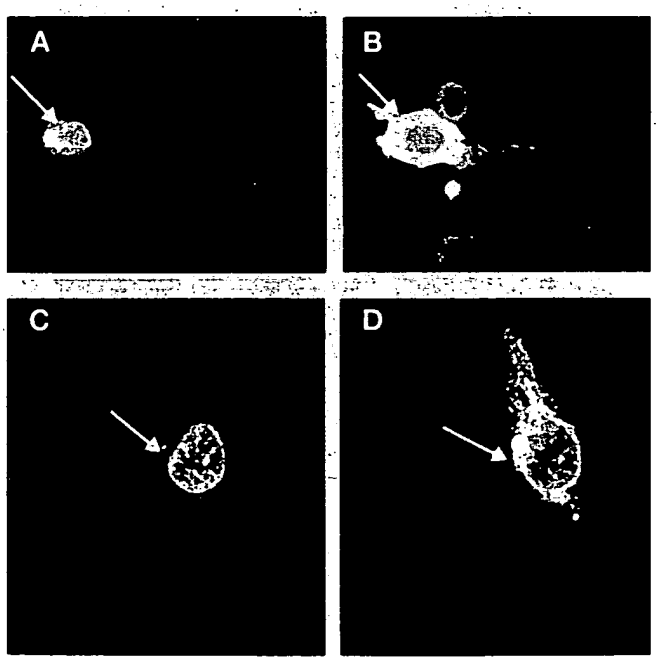

Figure 4. COS-1 cells 24 h posttransfection with (A,B) wild type VP22 or (C and D) VP22-p53 were co-plated with untransfected SAOS-2 cells at a ratio of 1:20 and after a further 20 h, double-labeled for (A and C) T-antigen and (B and D) VP22. Expressing T-antigen-positive COS-1 cells are labeled with an arrow.

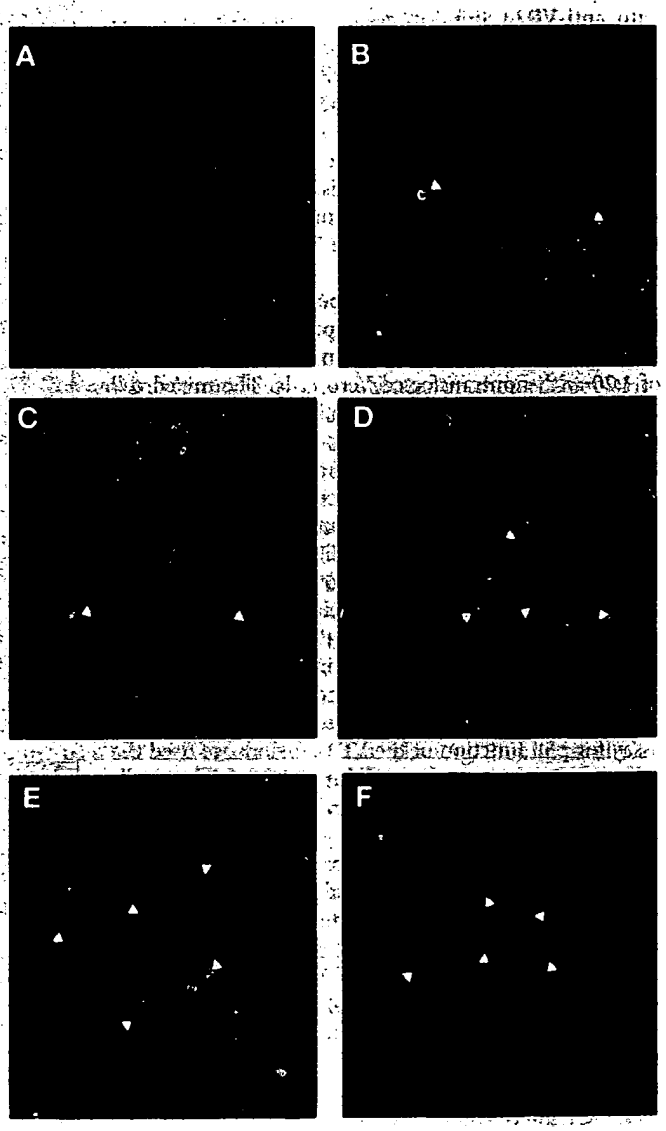

Figure 5. VP22-p53 and p53-VP22 can induce apoptosis in SAOS-2 cells. (A) Untransfected SAOS-2 cells or (B) cells 40 h posttransfection with pUC19, (C) wild type VP22, (D) wild type p53, (E) VP22-p53, or (F) p53-VP22 were labeled using the TUNEL method to detect apoptotic cells. The monolayers were counterstained with propidium iodide. Apoptotic TUNEL positive cells are indicated with arrows.

with *p53* and co-plated with SAOS cells demonstrated only p53-positive COS cells, with no spread into adjacent SAOS cells or cytotoxic effect on the SAOS cells (data not shown). However, when COS cells expressing VP22-p53 (Fig. 4C) were co-plated with untransfected SAOS-2 cells, we observed a cytotoxic effect such that surrounding cells were frequently lost, suggesting functional *p53* activity in neighboring recipient cells (Fig. 4D).

As a further direct test for function of the chimeric VP22-p53 proteins, induction of apoptosis in SAOS-2 cells was measured using the TUNEL assay (Fig. 5). Untransfected cells or cells 40 h posttransfection with pUC19, wild type VP22, wild type p53, VP22-p53, or p53-VP22 were labeled using the TUNEL method, and the monolayer was counterstained with propidium iodide to detect DNA. Transfection with pUC19 and wild type VP22 resulted in a low level of apoptosis in the SAOS-2 cells (Fig. 5B and C) representing 4% and 11% of the total monolayer respectively. An increase in the number of TUNEL positive cells was observed in the wild type *p53* transfected cells (Fig. 5D) with the figure increasing to 17%. However, a striking number of TUNEL positive cells was observed in both VP22-p53 (36%) and p53-VP22 (32%) transfected monolayers (Fig. 5E and F), with several apparently propidium iodide negative, TUNEL positive (green) cells at the advanced stages of apoptosis. Complete analysis of the numbers is hampered by the loss of SAOS cell monolayer in the VP22-p53 and p53-VP22 transfected cells, and thus the figure for the VP22 fusions is likely to be an underestimate. The high number of TUNEL positive cells and the widespread cytotoxic cells seen by crystal violet staining of the monolayer, was consistent with the fusion proteins inducing apoptosis in both expressing and recipient cells.

Exogenous addition of wild type *p53* into tumor cells can promote cell cycle arrest and/or the induction of apoptosis resulting in tumor regression, with examples including colorectal carcinoma[6], cervical cancer[7], and breast cancer[8,9]. A number of *p53* delivery systems including retroviral[10,11] or adenoviral vectors[7,12,13] and liposome systems[9], have been devised with reasonable success. Delivery of functional protein into a sufficiently high percentage of cells remains a major difficulty in this as in other similar strategies in gene therapy for the introduction of corrective genes for disease treatment. The VP22 fusion protein may be delivered by any of the above systems and the amplification of cell target number by intercellular transport of the protein synthesized in an initial subpopulation of cells is likely to prove of benefit in such therapeutic strategies. The ability to transport large multi-functional proteins may effect other aspects of gene therapy. For example, the bystander effect observed using enzyme-prodrug combinations for cancer therapy and thought to be due to metabolite transport[14] may be considerably enhanced by transport of the enzyme itself as a VP22 fusion. That a large fusion protein of approximately 90 Kda can be transported between cells and that this fusion protein retains the ability to induce apoptosis suggest that VP22 may prove of general utility in the delivery of other classes of protein, including transcription factors, enzymes, and antigens.

Experimental protocol

Plasmids. We constructed the plasmid pc49epB, which contains the VP22 open reading frame, in the background of pcDNAamp 1.1 (Invitrogen, NV Leek, Netherlands). The cytomegalovirus (CMV) promoter region and N-terminal region derive from pGE109 (ref. 15), such that the ATG start is immediately preceded by a unique BglII site. The C-terminus of VP22 derived from the plasmid pUL49ep[16] where the last residue is immediately preceded by a unique BamHI site and reads in frame to the epitope sequence ERKTPRVTGG derived from the *UL83* gene of HCMV[17]. This epitope is recognized by the monoclonal antibody CMV-018-48151 (Capricorn Products, Scarborough, ME). The coding region for *p53* was amplified by PCR using primers that contained either BglII or BamHI sites and cloned into the unique BglII or BamHI sites such that VP22, p53 and the epitope remained in frame for protein expression.

Antibodies. Polyclonal anti-VP22 antibody AGV30 (ref. 2) was used at a dilution of 1:10,000 for Western blot analysis and 1:500 for immunofluorescence. Monoclonal anti-p53 antibody DO-1 (Santa Cruz, Wembley, UK) was used at 1:1000 for Western blot analysis and 1:100 for immunofluorescence. Monoclonal anti-epitope antibody, which detects the CMV Late Nuclear Protein UL83 epitope (Capricorn Products) was used at 1:1000 for Western blot analysis and 1:100 for immunofluorescence. Monoclonal anti-T antigen pAB419 (ref. 18) was used at a dilution of 1:100 for immunofluorescence.

Transfection. For immunofluorescence and TUNEL assay, cells were plated at 2×10^5 cells per 35 mm dish and transfected with 1 μg of expression plasmid, made up to 2 μg with pUC19 DNA, using the calcium phosphate precipitation technique modified with BES-buffered saline.

Western blot analysis. Transfected cell monolayers were lysed in SDS sample buffer and proteins separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with appropriate primary antibody before detection of reactive bands using a horseradish peroxidase secondary antibody and ECL detection reagents according to the manufacturer's instructions (Amersham, Aylesbury, UK).

TUNEL assay. TUNEL labeling of transfected SAOS-2 cells was performed as described in the Appligene Oncor (Durham, UK) TUNEL kit manual.

Immunofluorescence and microscopy. Performed as described previously[2].

Acknowledgments

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